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Glycoprotein E (gE) specified by bovine herpesvirus type 5 (BHV-5) enables *trans*-neuronal virus spread and neurovirulence without being a structural component of enveloped virions

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Abstract

Bovine herpesvirus 5 (BHV-5) is a neurovirulent alpha-herpesvirus that causes fatal encephalitis in calves. We previously demonstrated that deletion of a glycine-rich epitope in the gE ectodomain dramatically reduced BHV-5 neurovirulence. To investigate the role of gE cytoplasmic tail sequences in the neuropathogenesis of BHV-5 in rabbits, we constructed a BHV-5gE recombinant virus with a short residual cytoplasmic domain lacking the YXXL motifs and the acidic (BHV-5gEAm480). *In vitro*, BHV-5gEAm480 produced on the average smaller plaques, compared with wild-type BHV-5, but it produced on the average substantially larger plaques than the gE ORF-deleted BHV-5. The truncated gE was not phosphorylated, and was not endocytosed from the cell surface. Importantly, the truncated gE was not incorporated into enveloped infectious virions, but its glycosylation and interaction with gI were not affected. In a rabbit model of infection, the BHV-5gEAm480 remained highly virulent, while the gE-null virus was avirulent. The gEAm480 mutant virus invaded most of the central nervous system (CNS) structures that are invaded by the wild-type BHV-5. The number of neurons infected by BHV-5gEAm480 was very similar to the number infected by BHV-5 wild-type and gEAm480-rescued viruses. Collectively, the results suggest that gE functions in transsynaptic transmission of BHV-5 and neurovirulence without being a structural component of the virion particle.

Keywords: BHV-5; Glycoprotein E; Neuropathogenesis

Introduction

Bovine herpesvirus type 5 (BHV-5) is an alpha-herpesvirus that causes fatal encephalitis in calves, and is a significant viral pathogen in South America (Belknap et al., 1994; D'Offay et al., 1993). Bovine herpesvirus type 1 (BHV-1) is associated with abortions, respiratory infections (subtype 1.1), and genital infections (subtype 1.2) in cattle (Wyler et al., 1989), but does not usually cause encephalitis. The BHV-1 and BHV-5 proteins have 82% predicted amino acid homology (Delhon et al., 2003), and both viruses establish latency in the trigeminal ganglion (TG) after intranasal and conjunctival inoculation (Ashbaugh et al., 1997; Rock et al., 1986). In a rabbit seizure model, BHV-1.1 and BHV-5 infections are distinguished by their differential neuropathogenesis (Chowdhury et al., 1997). When rabbits are inoculated intranasally, BHV-5 invades the central nervous system (CNS) via the olfactory pathway and produces acute neurological signs that are comparable to those seen in calves (Belknap et al., 1994). But BHV-1 does not invade the brain of infected rabbits, and neurological signs do not typically develop (Lee et al., 1999).

The gE/gI homologues in alpha-herpesviruses, including BHV-1 and BHV-5, form a noncovalently linked hetero-oligomer complex that is required for gE and gI maturation, cell to cell spread, and neurovirulence (Dingwell and Johnson, 1998; Enquist et al., 1999; Litwin et al., 1992; Mijnes et al., 1997; Whealy et al., 1993; Whitbeck et al., 1996; Zuckermann et al., 1988). In pseudorabiesvirus (PRV) and herpes simplex virus (HSV-1), gE or gI null mutants have significantly reduced neurovirulence, and their ability to infect second- and third-order neurons after nasopharyngeal or ocular infection is

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significantly reduced (Card et al., 1992; Dingwell et al., 1995; Enquist et al., 1999; Jacobs, 1994; Kritas et al., 1994, 1995; Mulder et al., 1994). In BHV-1, PRV and HSV-1, the respective gE ectodomains are sufficient for the complex formation with their counterpart gI (Mettenleiter, 2003; Rizvi and Raghavan, 2001; Tyborowska et al., 2000). In HSV-1, gE is important for the axonal localization of glycoproteins, viral capsids and VP22 tegument protein (Wang et al., 2005). Additionally, the gE ectodomain in PRV is more important than the gE cytoplasmic tail domain in axonal localization of capsids (Ch'ng and Enquist, 2005).

In our rabbit seizure model, gE-deleted BHV-5 has restricted anterograde transport from the olfactory receptor neurons to the bulb (second-order neurons) and does not infect second- and third-order neurons efficiently (Chowdhury et al., 2000). In the same model, BHV-1gE did not complement for the BHV-5gE with respect to neuroinvasion and neurovirulence (Chowdhury et al., 2000). Recently, we have determined that the role of gE in BHV-5 neurovirulence does not require the presence of gI (Al-Mubarak and Chowdhury, 2004). These results indicated that BHV-5gE plays an important role(s) in the differential neuropathogenesis of BHV-5.

Alignment of the predicted amino acid sequence of the *BHV-5gE* gene with the corresponding gE sequence of BHV-1 showed that BHV-5gE ectodomain contains a glycine-rich region (residues 204 to 218) that is significantly different from the corresponding gE region of BHV-1 (Chowdhury et al., 2000). We have recently determined that a glycine-rich epitope coding region of BHV-5gE is important for gE-mediated neurovirulence and anterograde neuronal transport in rabbits (Al-Mubarak et al., 2004). A BHV-5 with the glycine-rich gE epitope deleted formed wild-type-sized plaques in Madin-Darby bovine kidney (MDBK) cells; in vivo, however, the virus had a defective anterograde transport from the olfactory receptor neurons to the olfactory bulb, and its neuropathogenicity was reduced significantly (Al-Mubarak et al., 2004).

Based on the sequence alignment (Chowdhury et al., 2000), there are two YXXL potential tyrosine kinase phosphorylation motifs within the gE cytoplasmic tail (Y is a tyrosine, X is any amino acid, and L is a lysine and represents a hydrophobic residue) that are conserved in the two gE sequences (Chowdhury et al., 1997). There are some differences between their acidic domain sequences downstream of the YXXL motif (Chowdhury et al., 2000). In HSV-1, PRV, and Vericella–Zoster virus (VZV), the YXXL motifs within their gE cytoplasmic tails are also important for endocytosis and/or gE recycling from the plasma membrane to the *trans*-Golgi network and for incorporation of gE in the virion envelope (Olson and Grose, 1997; Tirabassi and Enquist, 1999; Tirabassi et al., 1997). In PRV, the mutation within the tyrosine kinase YXXL motifs resulted in slightly reduced neurovirulence in rat (Tirabassi and Enquist, 1999).

In this study, the objective was to construct a BHV-5 with a truncated gE cytoplasmic tail (BHV-5gEAm480), and then determine the functional significance of these sequence differences in the rabbit model of BHV-5 neuropathogenesis. In addition, the role of the gE cytoplasmic tail sequence in gE processing, gE endocytosis and gE incorporation into virion envelope, and cell to cell spread on MDBK cells was determined.

Results

Construction and analysis of gE-deleted, gEAm480, and gEAm480R BHV-5 viruses

A gE-deleted BHV-5 recombinant virus was constructed, previously, in which the entire gE cytoplasmic domain coding region was intact (Chowdhury et al., 2000). Therefore, a BHV-5 with the entire gE ORF deleted was constructed. To construct a BHV-5 with gE cytoplasmic tail sequences including the YXXL motifs plus the acidic domain sequences truncated (BHV-5gEAm480), we rescued the gE-deleted BHV-5 by a plasmid, pBHV-5gEAm480 coding for a truncated gE in which expression of gE amino acid residues 480 to 598 was terminated. Several non-fluorescent recombinant virus plaques were plaque purified, and the gE sequence of BHV-5gEAm480 was verified by sequencing for the incorporation of the amber mutation. One BHV-5gEAm480-rescued virus (BHV-5gEAm480R) was generated and verified by immunoblotting and immunoprecipitation (see below).

To verify the gEAm480 expression, infected cell lysates from BHV-5gEAm480, BHV-5gEAm480R and BHV-5 wild-type viruses were analyzed by immunoblotting with gE-specific polyclonal goat serum (Fig. 1A). The antibody recognized two gE-specific bands in the BHV-5gEAm480R and wild-type lysates. These are the ER-processed, 86-kDa, and the Golgi processed, 94-kDa mature, gE proteins. These two bands were absent in the lysates of BHV-5gEΔ EGFP virus. In the lane containing lysate of BHV-5gEAm480, the antibody recognized two bands with approximate molecular mass of 70-kDa (ER processed) and 81-kDa (Golgi processed). To determine whether the cytoplasmic tail truncated version of gE (gEAm480) and the gI expressed by BHV-5gEAm480 virus were incorporated in the virion envelope, immunoblotting analyses of partially purified virion lysates with gE-, gI- and gC-specific antibodies were performed. The results show that when 2- to 3-fold more protein is loaded in the lane containing the gEAm480 virion lysate, based on gC-specific band (Fig. 1C), gEAm480-specific band was barely detectable (Fig. 1B). Interestingly, incorporation of proteolytically processed mature 45-kDa gI^C band (Al-Mubarak and Chowdhury, 2004) in the virion was detectable but the amount was reduced relative to the wild-type virions, significantly. Notably, the 62-kDa mature BHV-5 gI band was not detectable by immunoblotting when 2- to 3-fold protein is loaded in the case of BHV-5gEAm480 virions (Fig. 1B). Based on these results, we believe that the gE cytoplasmic tail is necessary for efficient incorporation of gE and gI in the virion envelope.

Maturation of mutant gE (Am480) and gEAm480-gI interaction

Immunoprecipitation and pulse-chase analysis of wild-type BHV-5 and BHV-5gEAm480 virus-infected cell proteins with

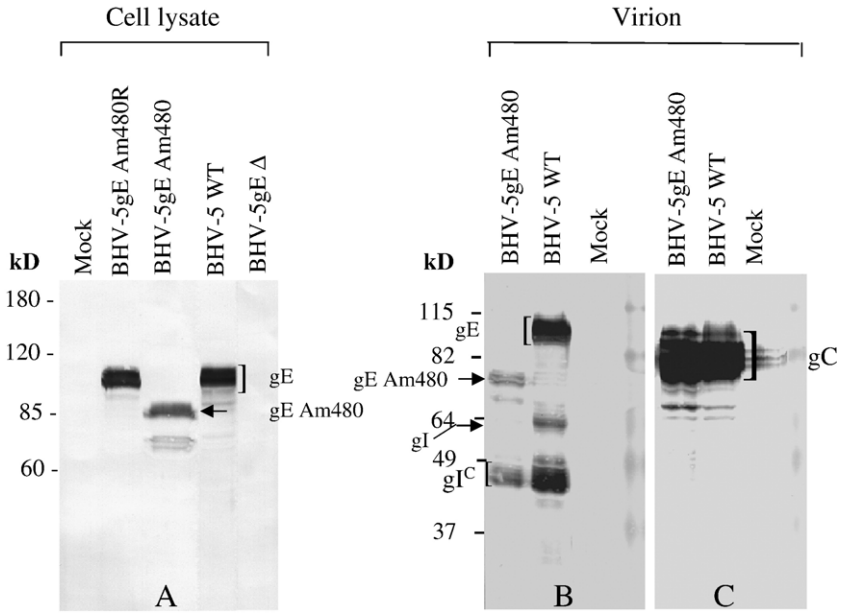

Fig. 1. Identification and characterization of BHV-5gEAm480 virus. (A) Immunoblot analysis of cell lysates with goat gE-specific antibody. Cells were either mock-infected or with BHV-5gEAm480-rescued (BHV-5gEAm480R), BHV-5gEAm480, wild-type BHV-5, and BHV-5gE-deleted viruses. (B) Immunoblot analysis of purified BHV-5gEAm480 virion, BHV-5 WT virion and mock-infected MDBK cell lysates with a cocktail of rabbit gE and gI-specific polyclonal antibodies. Note that the amount of BHV-5gEAm480 virion lysates loaded was 2–3 times more relative to BHV-5 wild type (based on the amount of gC, see below). (C) Immunoblot analysis of identical blots with gC-specific monoclonal antibody (Chowdhury, 1995).

gE-specific polyclonal goat serum showed that the cytoplasmic tail truncated gE processing was similar to the wild-type gE. The dominant gE bands observed immediately after the pulse (0-min sample) were 86-kDa ER-processed wild-type gE and 70-kDa ER-processed cytoplasmic tail-truncated gEAm480 and both were EndoH sensitive (Fig. 2A). By 240-min chase, both the 86-kDa (wild-type gE) and 70-kDa (gEAm480) bands were processed in the Golgi to mature 94- and 85-kDa bands, respectively and they became EndoH resistant. (Fig. 2A). As expected, both the wild-type and mutant (gEAm480) EndoH-resistant gE bands were sensitive to glycopeptides F (data not shown).

The results also show that immediately after 30-min pulse labeling (0-min sample), 46-kDa ER-processed, gI-specific bands are coimmunoprecipitated by a goat gE-specific antibody from both the wild-type and mutant viruses-infected cell lysates (Fig. 2A). After a 240-min chase, the 62-kDa and 45-kDa mature and proteolytically cleaved gI^C (proteolytically processed from the mature gI) bands, respectively (Al-Mubarak and Chowdhury, 2004) are coprecipitated with gE-specific antibody. While the 46-kDa (ER-processed gI) bands in both cases were EndoH sensitive, the 62-kDa (mature) and 45-kDa (gI^C) bands were resistant to EndoH (Fig. 2A).

To demonstrate that the 62-kDa and 45-kDa bands coimmunoprecipitated by the goat gE-specifc antibody are indeed mature and proteolytically cleaved gI-specific bands, respectively, immunoblotting analysis of coimmunoprecipitated proteins were performed with either a rabbit gE-specifc (Fig. 2B) or a cocktail of gE- and gI-specific antibodies (Fig. 2C) (Whitbeck et al., 1996; kindly provided by Dr. L. Enquist, Princeton University). As expected, these bands were specifically recognized by the gI-specific antibody (Fig. 2C).

Taken together, these results indicated that gE of BHV-5gEAm480 formed a complex with the gI in the endoplasmic reticulum, was transported to Golgi, and was processed to its mature form within the Golgi apparatus.

gE phosphorylation

The cytoplasmic tail of BHV-5gE contains three YXXL tyrosine kinase phosphorylation motifs. To determine the gE phosphorylation in BHV-5 wild-type and BHV-5gEAm480, virus-infected cells were labeled with ^{32}P. Mock- and virus-infected ^{32}P-labeled lysates were immunoprecipitated with gE-specific polyclonal goat serum. The immunoprecipitated proteins were separated by SDS-PAGE, and the Western blot was analyzed by using gE-specific polyclonal rabbit serum to eliminate unwanted heavy and light chain bands from the goat IgG polyclonal antibody. ^{32}P-labeled (phosphorylated) and immunoprecipitated gE-specific proteins (gE wild type and gEAm480) were visualized by autoradiography. The autoradiograph of the immunoblot demonstrated that both gE forms, wild-type and gEAm480, were precipitated by anti-gE antibody but only the wild-type gE protein was phosphorylated at detectable levels (data not shown).

Growth characteristics and plaque size of BHV-5gEAm480 virus in MDBK cells

To determine the one-step growth property of BHV-5gEAm480 virus relative to the wild type, MDBK cells

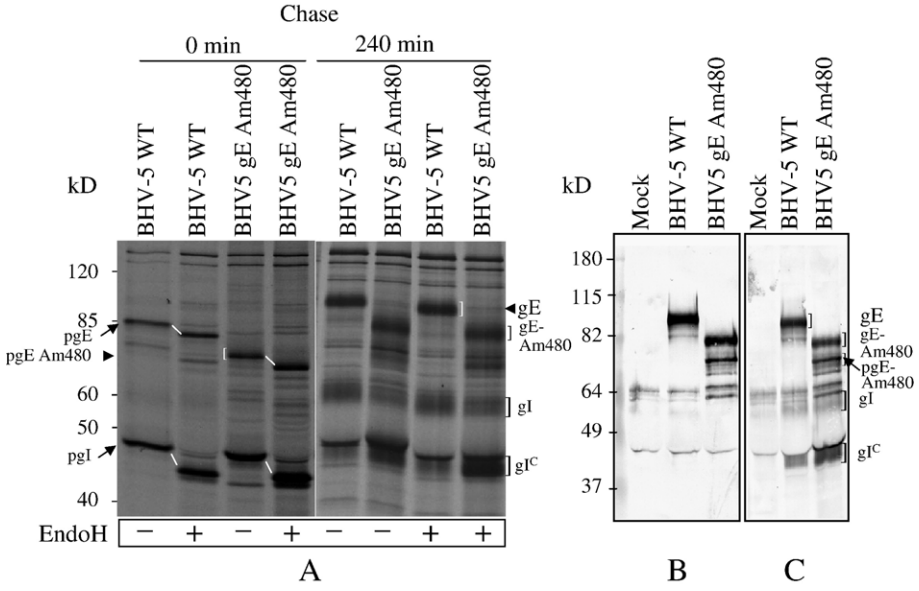

Fig. 2. Analysis of gE/gI processing and gE/gI interaction in BHV-5 wild-type and BHV-5 gEAm480 by immunoprecipitation. For gE/gI interaction and processing, infected MDBK cells were pulse-labeled for 30 min in 100 μCi of [^{35}S] methionine-cysteine per milliliter beginning at 6 h post-infection. Cell monolayers were the washed twice with serum-free DMEM and incubated in complete growth medium without labeled cysteine and methionine. Detergent extracts were prepared as described earlier at different times post pulse labeling (0-min and 240-min chase are shown). Following immunoprecipitation with gE-specific polyclonal goat serum, EndoH digestions were performed as previously (Al-Mubarak et al., 2004). Immunoprecipitated proteins were separated by SDS-PAGE (10%) and analyzed by autoradiography (A). To verify the specificity of immunoprecipitated bands further, cold infected cell lysates were immunoprecipitated with gE-specific polyclonal goat serum in duplicate. Western blots containing identical immunoprecipitates were either immunoblotted with rabbit gE-specific polyclonal antibody (B) or with cocktail of gE and gI-specific rabbit polyclonal antibodies (C).

were infected with respective virus as described previously (Chowdhury et al., 2000). Virus titration results up to 42 h post-infection (data not shown) show that growth kinetics of the BHV-5gEAm480 relative to BHV-5gEAm480R and BHV-5 wild-type are very similar.

To compare the plaque size of BHV-5gEAm480 to BHV-5gEAm480R and BHV-5gE-deleted viruses, MDBK cell monolayers were infected with these viruses and overlaid with a medium that contained 1.6% carboxymethylcellulose. The infected cells were fixed at 48 h post-infection and were stained with crystal violet stain. The BHV-5gEAm480 virus produced on the average smaller plaques relative to those of BHV-5gEAm480R. However, BHV-5gEAm480 virus produced on the average substantially larger plaques compared to that of gE ORF-deleted (BHV-5gEΔ) BHV-5 (Fig. 3).

Endocytosis of gE proteins

The mature gE of alpha-herpesviruses is recycled between the *trans*-Golgi network and cell surface of infected cells (Tirabassi and Enquist, 1998). To investigate the role of the BHV-5gE cytoplasmic tail domain in the gE endocytosis after cell surface expression, an indirect immunofluorescence endocytosis assay was performed as described in the Materials and methods. As shown in Fig. 4, gE was detected on the cell surface of infected cells at 0 min for both the wild-type and gEAm480 when the cells were fixed immediately after incubation with the anti-gE antibody at 4 °C and were not incubated at 37 °C. When cells were incubated further, for 15 or 30 min at 37 °C, the wild-type gE protein was internalized and accumulated in the interior of the cells; but the gEAm480 failed to internalize. After 15- or 30-min incubation at 37 °C, the gEAm480 was still detected on the cell surface.

Pathogenicity of BHV-5gEAm480 and BHV-5gEAm480R viruses in rabbits

Two groups of twenty rabbits, each, were either inoculated with BHV-5gEAm480 or with gEAm480-rescued BHV-5 and ten rabbits (3rd group) were inoculated with wild-type BHV-5. By 10–11 dpi, six out of 20 rabbits (30%) infected with BHV-5gEAm480 showed severe neurological signs and one rabbit (5%) showed mild neurological signs. Whereas 9 out of 20 (45%) and 6 out of 10 (60%) rabbits infected with BHV-5gEAm480 rescue and BHV-5 wild type, respectively showed severe neurological signs (Table 1). The rabbits were euthanized at 12 dpi or when they showed severe neurological signs. Three rabbits, in each group (gEAm480 or gEAm480 rescue) showing severe neurological signs were processed for virus isolation or for immunohistochemistry as described in Materials and methods. A summary of virus isolation and virus spread (immunohistochemistry) results is shown in Tables 1 and 2, respectively. Relative to BHV-5 wild-type, amount of virus isolated from the nasal swabs of rabbits infected with BHV-5gEAm480 was similar (Table 1) indicating that the mutant virus replicated efficiently in the naso/olfactory epithelium. Compared to BHV-5gEAm480 virus-infected rabbits, slightly more virus was isolated from brain

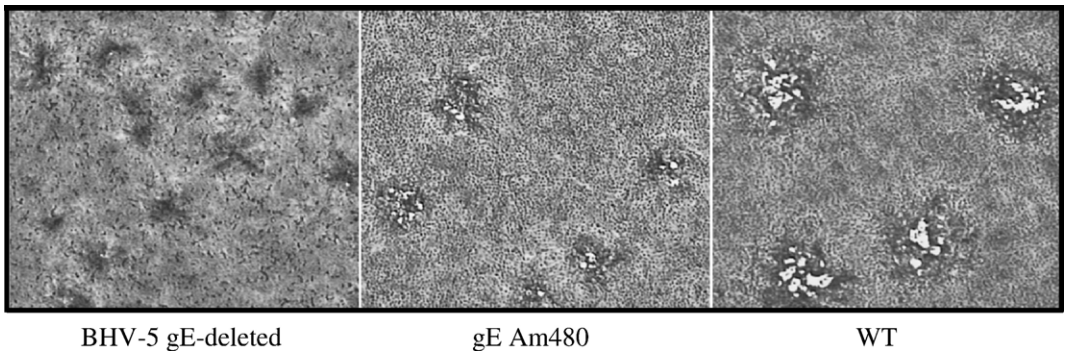

Fig. 3. Comparative plaque morphology of BHV-5gEAm480, BHV-5 WT, and BHV-5gEΔ viruses. MDBK cells were infected with the different viruses and at 2 h post-infection, the medium was replaced with 1.6% carboxymethylcellulose. At 48 h post-infection, cells were fixed and stained with 0.35% crystal violet solution. Entire field at 4× magnification is shown for each viruses.

segments of rabbits infected with the BHV-5gE-rescued virus. Immunohistochemistry results showed that, in rabbits infected with BHV-5gEAm480, virus-specific antigen was detected in infected neurons of most of the olfactory tract areas infected by the wild-type or BHV-5gEAm480-rescued viruses (Fig. 5). In general, the number of neurons infected by BHV-5gEAm480 was slightly reduced, compared with the wild-type or the gEAm480-rescued virus (Fig. 5). To verify that viruses isolated from the brains of rabbits infected with BHV-5gEAm480 virus expressed the truncated version of the gE, immunoblotting analysis of a BHV-5gEAm480 brain isolate was performed. The results shown in Fig. 6 confirmed that the

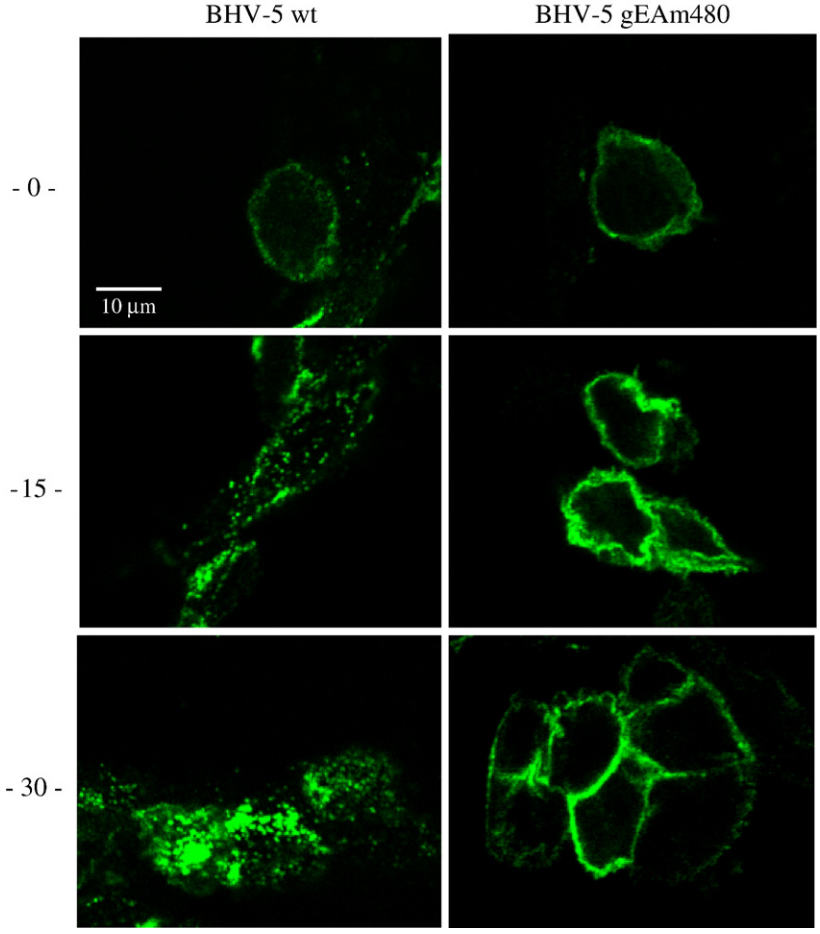

Fig. 4. Endocytosis of gE in cells infected with wild-type and BHV-5gEAm480 viruses. MDBK cells were infected with wild-type and BHV-5gE Am480 at 5 PFU per cell for 8 h before an indirect immunofluorescence endocytosis assay as described in Materials and methods. Briefly, the cells were incubated at 4 °C with gE-specific polyclonal goat serum for 30 min on ice before being shifted to 37 °C for the indicated times to allow internalization of cell surface proteins with bound primary antibody. The cells were then fixed, permeabilized, and incubated with an Alexa-488-congugated secondary antibody to detect bound primary antibody.

Table 1
Summary of clinical signs and virus isolation

Virus	Animal no.	Neurological signs[a]	PFU[b]	Virus isolation[c]		
			Nose 3 dpi	Olfactory bulb	Anterior cortex	Posterior cortex
BHV-5 Am480	20	Severe[d] (6) Mild (1)	1.0×10^3	–	100	95
BHV-5 Am480R	20	Severe[e] (9)	2.0×10^3	40	300	270
BHV-5 (wt)	10	Severe[f] (6)	1.2×10^3	200	900	800

[a] Numbers in parenthesis indicate number of animals showing clinical signs or brain segments positive for virus isolation.
[b] Average PFU.
[c] Average PFU/g of tissue.
[d] Results are from two experiments; in the first and second experiments 2 and 4 rabbits out of 5 and 15 rabbits, respectively (total 6 out of 20) showed severe neurological signs.
[e] Results are from two experiments; in the second and 3rd experiments 4 and 5 rabbits out of 10 rabbits, respectively (total 9 out of 20) showed severe neurological signs.
[f] Results are from two experiments; 3 rabbits out of 4 (1st experiment) and 3 rabbits out of 6 (3rd experiment), total 6 out of 10 showed severe neurological signs.

brain isolates expressed the truncated version of the gE. Taken together, the results suggest that BHV-5gEAm480 virus invaded 4th- and 5th-order olfactory neuronal connections, and the CNS spread was similar to that of the wild-type or BHV-5gEAm480-rescued viruses. Therefore, BHV-5gEAm480 retained significant neuroinvasiveness and neurovirulence in rabbits.

Discussion

We reported previously that BHV-5gE is important for neurovirulence and neural spread in rabbits after intranasal infection (Chowdhury et al., 2000). Specifically, we showed that a glycine-rich epitope coding region within the BHV-5gE ectodomain is important for BHV-5 neurovirulence and neural spread (Al-Mubarak et al., 2004). In this study, we focused on the potential role of the cytoplasmic domains of gE in neuronal spread and neurovirulence. The salient features of our investigations are: (1) truncation of the entire cytoplasmic domain of gE leads to lack of gE incorporation into enveloped infectious BHV-5 virions; (2) the BHV-5 mutant virus gEAm480 specifying the carboxyl terminal truncation of gE retains nearly wild-type levels of neuronal spread and neurovirulence in vivo. These results show that the cytoplasmic tail of gE is not important for neuronal spread and neurovirulence. Importantly, the results are consistent with the assumption that the truncated gE expressed on cell surfaces and probably on the synaptic membranes may function in virus spread without being a structural component of the virion particle.

Like the wild-type gE, cytoplasmic tail truncated gE (gEAm480) was processed in the Golgi, formed a complex with the gI, and reached the cell surface. Therefore, consistent with previous reports in other alpha herpesviruses (Mettenleiter, 2003; Rizvi and Raghavan, 2001; Tyborowska et al., 2000), BHV-5gE cytoplasmic tail is not important for complex formation with the gI, gE/gI processing and maturation within the Golgi. Unlike the wild-type gE, the gEAm480 was not phosphorylated, it was not endocytosed and it did not get incorporated into enveloped virions. In several herpesviruses, including HSV, PRV and VZV, the YXXL motifs located within the gE cytoplasmic tail are important for tyrosine kinase phosphorylation and gE endocytosis from the cell surface (Olson and Grose, 1997; Tirabassi and Enquist, 1998; Tirabassi et al., 1997). Therefore, the lack of both gEAm480 phosphorylation and gEAm480 endocytosis was expected. Clearly, deletion of the cytoplasmic domain of BHV-5 resulted in lack of gE incorporation into virion particles. Similar results have been found with PRV (Tirabassi et al., 1997; Tirabassi and Enquist, 1998). Specifically, in the case of PRV, incorporation of gE in the PRV envelope required the YXXL motif within the gE cytoplasmic tail (Tirabassi et al., 1997; Tirabassi and Enquist, 1998). However, HSV-1 mutant viruses without the entire gE cytoplasmic tail, including the YXXL motif, incorporated the mutant gE on the virion envelope (Farnsworth and Johnson, 2006). Therefore, signals for gE incorporation must be substantially different between HSV-1 and those of BHV-5 and PRV.

In vitro, BHV-5gEAm480 virus produced on the average smaller plaques when compared with those of BHV-5gE Am480-rescued virus and BHV-5 wild-type viruses, but it produced on the average substantially larger plaques than the gE ORF-deleted BHV-5. Therefore, the gE ectodomain and/or gE transmembrane domain must facilitate cell to cell virus spread in cell culture in the absence of the carboxyl terminus of gE. Similar findings were also reported for HSV-1 and PRV

Table 2
Summary of viral spread in the brain after intranasal inoculation

Virus	Pres[a]				
	AON	PIR	HIPPO	AMYG	LC
BHV-5 gEAm480	757	777	109	2700	24
BHV-5 gEAm480R	1021	533	350	625	8
BHV-5 (wt)	1155	788	228	1100	25

AON, anterior olfactory nucleus; PIR, piriform cortex; HIPPO, hippocampus; AMYG, amygdala; LC, locus coeruleus; scores for the BHV-5 gEAm480, BHV-5 gE Am480 rescue (BHV-5gEAm480R) and BHV-5 wild-type are average of 2 rabbits and for each segment at least 10 sections were analyzed. Neurons labeled in the AON, PIR, AMYG are 3rd-order neurons while the labeled neurons in the HIPPO and LC are the 4th-order neurons.
[a] Labeled neurons per field at a magnification of ×5.

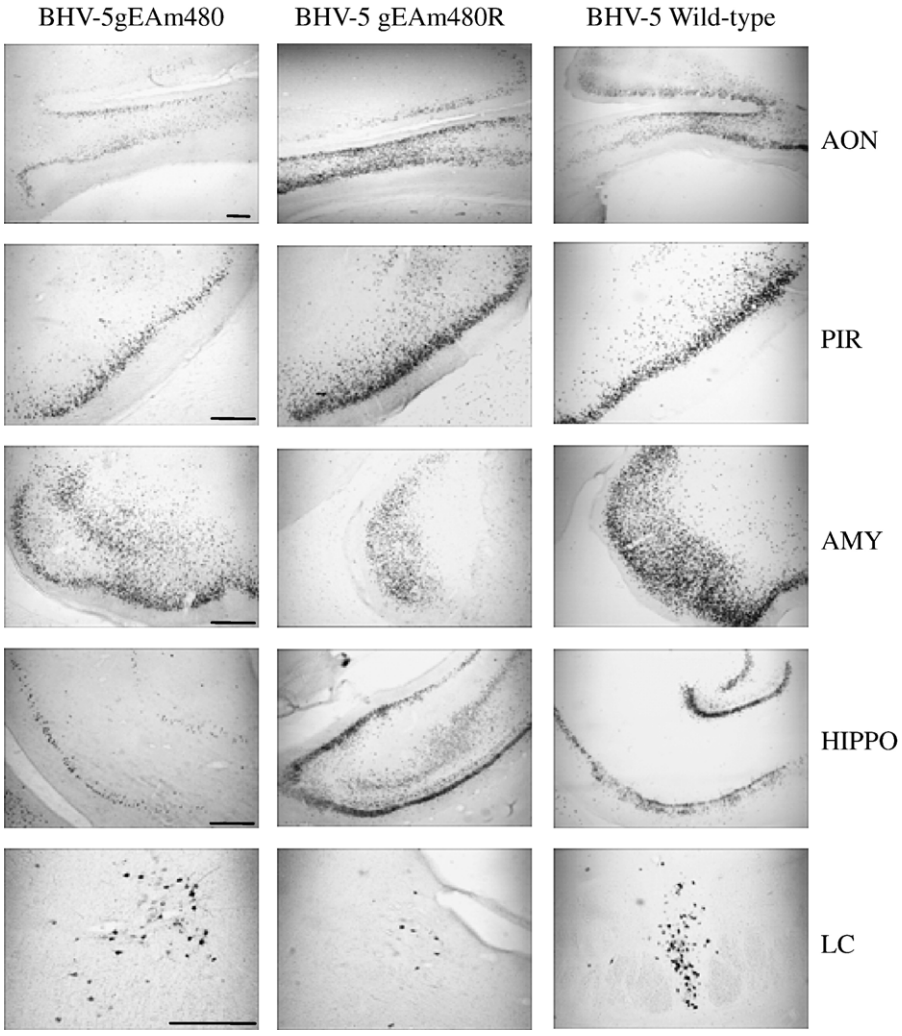

Fig. 5. Localization of viral antigen in brain sections. Animals were inoculated intranasally with either BHV-5gEAm480, BHV-5gEAm480R or BHV-5 wild type as described in Materials and methods. The animals were euthanized on day 11 day post-infection or when they showed neurological signs. The brains were processed for immunohistochemical analysis as described in Materials and methods. Representative sections of the olfactory bulb (OB), anterior olfactory nucleus (AON), piriform cortex (PIR), amygdala (AMYG), hippocampus (HIPPO), and locus coeruleus (LC) are shown.

(McMillan and Johnson, 2001; Tirabassi et al., 1997). Since the gE cytoplasmic tail domain in alpha herpesviruses may target the nascent virions to cell junction and redistribute gE in the cell junctions *in vitro* (Farnsworth and Johnson, 2006; Johnson et al., 2001), the lack of gE cytoplasmic tail could have contributed to its smaller size plaque relative to the wild type. Consequently, this function of gE, *in vitro*, is conserved in most alpha herpesviruses. Apparently, gE functions in virion spread must be independent of incorporation of gE into virion particles, since the gEAm480 mutant failed to incorporate gE into virion particle. Additionally, the role of gE cytoplasmic tail in the anterograde transsynaptic spread is different from its role in targeting nascent virions to cell junction *in vitro* in MDBK cells.

In rabbits, BHV-5gE Am480 had wild-type level neuroinvasiveness but slightly reduced neurovirulence relative to the gE cytoplasmic tail-rescued and wild-type BHV-5. There was a massive neuroinvasion of BHV-5gEAm480 virus in the 3rd- and 4th-order neurons of the olfactory pathway and 35% showed neurological signs (30% severe and 5% mild

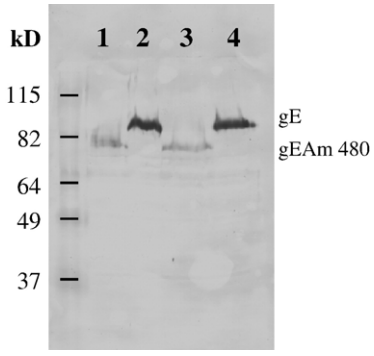

Fig. 6. Immunoblotting analysis of a brain isolate from rabbits infected with BHV-5gEAm480. MDBK cells were infected with: lane 1, BHV-5gEAm480; lane 2, BHV-5 wild type; lane 3, brain isolate from a rabbit infected with BHV-5gEAm480 and lane 4, BHV-5gEAm480R (rescued). Immunoblotting with gE-specific antibody was performed as above in Fig. 2.

neurological signs). This is not significantly different to the 45% and 60% of rabbits infected with gE-rescued and wild-type virus, respectively showing severe neurological signs. The data suggest that the gE cytoplasmic tail including the tyrosine kinase phosphorylation motifs and the acidic domain sequences do not play a significant role in the anterograde neuronal transport of BHV-5. Importantly, the data also suggest that gE can function in transsynaptic anterograde transmission of the virus without gE being a structural component of egressing virus. Until recently, it was well accepted that during anterograde axonal transport, viral envelope proteins and tegumented capsids are transported separately (Mettenleiter, 2003; Tomishima and Enquist, 2001). Recent findings in PRV raised the possibility that transport of fully enveloped virus particles or vesicles containing tegumented capsids are transported anterogradely within the axons (Antinone and Smith, in press).

The results presented in this manuscript support the hypothesis that BHV-1 gE ectodomain, lacking the entire carboxyl terminus serves critical functions in transsynaptic transmission of the virus. In PRV, the gE ectodomain is more important than the gE cytoplasmic tail domain in axonal localization of capsids and glycoproteins (Ch'ng and Enquist, 2005). This result is difficult to reconcile with a potential role of gE in the trafficking of vesicles containing either capsids or enveloped virions. The orientation of gE as well as all other viral glycoproteins within vesicle membranes must be with the extracellular portion oriented toward the lumen (outside) side and the carboxyl termini oriented toward the cytoplasm (inside). Therefore, the fact that the absence of the gE cytoplasmic domain does not prevent virus spread argues against any major contribution from the gE carboxyl terminus in targeting vesicular trafficking of virions to the plasma membrane and synapses.

Overall the results are consistent with the notion that gE expressed on cell surfaces (at the cellular junctions) and synaptic membranes can facilitate virion spread (Polcicova et al., 2005). It is possible that gE functions to modulate virion transport via localized breakdown of membrane barriers creating either localized membrane fusion domains and/or pore structures that allow either capsids or enveloped virions to spread. In this regard, gE may function in conjunction with other viral glycoproteins as well as specific cellular receptors that facilitate pore formation and virion transport.

Materials and methods

Virus strains and cell lines

The BHV-5 TX-89 strain (Chowdhury et al., 1997) was used in this study. The virus was propagated and titrated in MDBK cells grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum.

Generation of a BHV-5gE-specific antiserum

The plasmid pGST-gE5 was created as an expression vector for the production of polyclonal antiserum specific to BHV-5gE. The coding sequence for amino acid residues 144 to 329 of BHV-5gE was amplified by long PCR, using a forward primer that introduced a *Bam*HI site directly upstream of amino acid residue 144 and a reverse primer that introduced an *Eco*RI site directly downstream of amino acid residue 329. The 576-bp *Bam*HI-*Eco*RI PCR-generated fragment containing the coding sequence for amino acid residues 144 to 329 of BHV-5gE was then ligated in frame into the corresponding sites of the pGEX-4T-2 (Amersham Biotech), generating the pGST-gE5 vector.

Expression of the GST-gE5 fusion protein was induced by the addition of IPTG (isopropyl-Â-D-thiogalactopyranoside) to an *Escherichia coli* BL21 culture containing pGST-gE5 expression vector. The GST-gE5 fusion protein was 100% insoluble and was retained within the inclusion bodies. The fusion protein was solubilized by using 0.5% *N*-lauroylsarcosine (Sigma) and 0.5% Triton-X (Fisher) as described by Mercado-Pimental et al. (2002). The soluble, non-denatured fusion protein was then bound to glutathione–sepharose beads, and the recombinant gE protein was cleaved away from the GST tag by using thrombin protease according to the manufacturer's protocol (Amersham Biotech). The solubilized gE peptide was isolated from GST and used to immunize goats and rabbits (Cocalico Biologicals).

Virus purification

For virus purification, cells were scraped off when the 80–90% cells showed cytopathic effects. Pooled supernatants (supernatants following centrifugation at 2700×*g* for 10 min were ultracentrifuged for 2 h, at 112,700×*g* in a Beckman SW 28 rotor, through a sucrose (30% w/v) cushion which filled about 1/4th of the tube.

Western blot analysis

Cellular extracts and purified virion lysates were electrophoresed on a 10% SDS polyacrylamide gel and transferred to nitrocellulose membrane. Proteins were visualized by immunoblotting (Chowdhury et al., 1999) using gE-specific polyclonal rabbit or goat serum.

Construction of a BHV-5 with gE open reading frame (ORF) deleted

For the construction of BHV-5 recombinant virus with a truncated gE cytoplasmic tail, it was necessary to generate a BHV-5 with the entire gE open reading frame (ORF) deleted. For this, the gE upstream and downstream flanking regions were generated by long PCR as described before (Al-Mubarak et al., 2004). Using primer pairs P1-F/P2-R and P3-F/P4-R, and pKS95-15 DNA as a template, 1.14-kb and 0.9-kb fragments, respectively, were amplified (Table 3). These primer pairs incorporated *Eco*RI-*Kpn*I (P1 and P2, respectively) and *Bam*HI-*Hin*dIII (P3 and P4, respectively) at the 5′ and 3′ ends of the respective amplified fragment (underlined sequences represent the restriction sites). After PCR amplification, the P1–P2 and P3–P4 products were digested with *Eco*RI-*Kpn*I and *Bam*HI-

Table 3
Oligonucleotide primers

PCR products	Primer#	Nucleotide sequence (5′ to 3′)
gEΔ	P1 F	CGACGAATTCCGCTCCTGCCTGCAC
	P2 R	AGACGGTACCCTCAGTTCCTCTCGC
	P3 F	AAGTGGATCCTCCGCTAGGCGCCCC
	P4 R	CCGGAAGCTTGGGCGAGGGAAGAGA
gE Am480	P5 F	GGCCGCGTTTGTGCACGAATTCGACGGGCCCGGCTCCGACGA
	P6 R	GTCGTAGGTGCGCTGCTAGCCCGCGCGGCGCGCGCACACCCAGAC
	P7 F	GCGCGCGCCGCGCGGGCTAGCAGCGCACCTACGACATCCTCAAC
	P8 R	GCGGGGCGGAGGGGGGAAGCTTGGCGGAGGGGGAAAGAGAGT
gE Am480R*	P9 F	CCTGGGACTACAGCCCTGGTCGTCACCTC
	P10 R	CGGCAACCGGCACGTCTTCGATGGTGAGC

*P9 and P10 were only used for amplifying a 480-bp fragment to identify BHV-5 gEAm480-rescued virus-specific sequences.

*Hin*dIII enzymes, respectively, and cloned into the *Eco*RI-*Kpn*I and *Bam*HI-*Hin*dIII sites of plasmid pGEM-3Z (Promega, Madison, WI). The resulting clone (pBHV-5gEΔ 5′3′) lacked the entire gE ORF, but it contained the gE upstream gI sequences and gE downstream Us9-flanking sequences located immediately adjacent to the *Kpn*I and *Bam*HI sites, respectively.

To insert the *EGFP* gene cassette, a 1981-bp fragment containing the CMV immediate early promoter sequence, EGFP ORF sequence and SV40 poly A sequences were amplified by long PCR as described earlier (Chowdhury et al., 2006), and cloned into the *Kpn*I/*Hin*dIII sites of BHV-5gE deletion plasmid pBHV-5gEΔ 5′3′ constructed earlier. The resulting plasmid, pBHV-5gEΔ EGFP, lacks the entire gE ORF, and the *GFP* gene is flanked by the gE upstream and downstream sequences.

BHV-5gEΔ EGFP recombinant virus was generated by cotransfection of full-length wild-type BHV-5 and linearized pBHV-5gEΔ EGFP DNA in MDBK cells as described earlier (Chowdhury et al., 1999). Plaque purified recombinant viruses were further analyzed by immunoblotting with the gE-specific antibody.

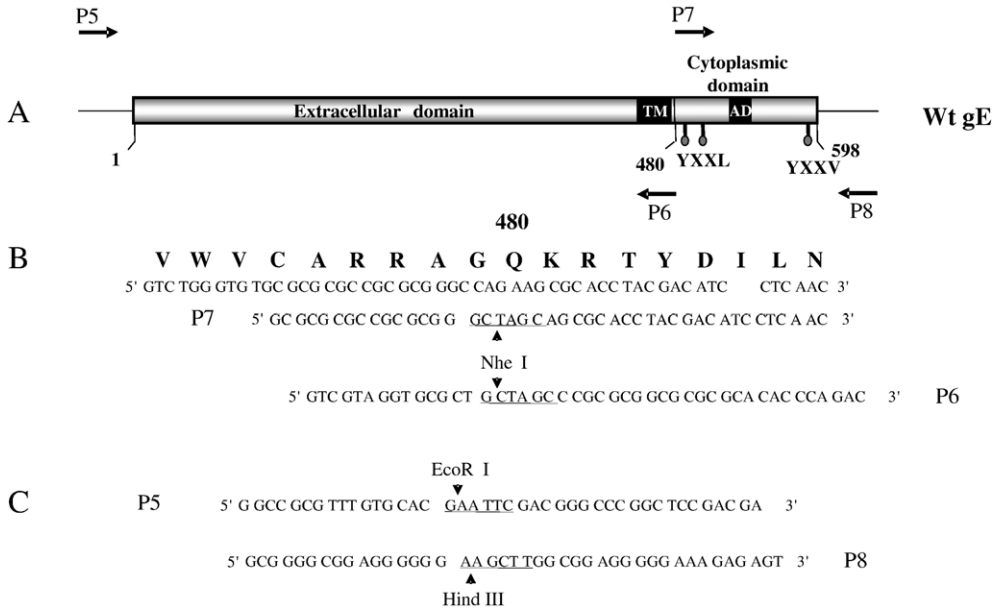

Fig. 7. Schematic illustration of the construction of BHV-5gEAm480. (A) Schematic of the *BHV-5gE* gene with the location of YXXØ motifs and acidic domain (AD) location within the cytoplasmic domain, is shown. The position of primer pairs P5–P6 and P7–P8 are indicated. (B) Scheme of the construction of the BHV-5 Am480 plasmid. In plasmid pBHV-5Am480, restriction sites for *Nhe*I created by primers P6 and P7. (C) Primer P5 and 8 sequences, used with P6 and P7, respectively, are shown with restricted sites created.

Construction of BHV-5gE Amber 480 recombinant virus.

The *Sma*I site in the multiple cloning site of the plasmid, pGEM-7Zf (Promega, Madison, WI), was changed to *Nhe*I site by linker ligation, resulting in the plasmid pKS02-02. To engineer a chimeric *gE* gene containing a gE Amber 480 (Am480) mutation, pgEAm480 recombinant vector was constructed. For this, DNA sequences upstream of the gE residue 481 were generated by long PCR with primer pair P5-F/P6-R (Fig. 7, Table 3). The primer pair incorporated *Eco*RI-*Nhe*I restriction sites (P5 and P6, respectively) at the 5′ and 3′ ends of the respective amplified fragment (underlined sequences represent the restriction sites). To amplify the DNA sequence downstream of gE residue 479, the primer pair P7-F/P8-R was used (Table 3). The primer pair incorporated *Nhe*I-*Hin*dIII restriction sites (P7 and P8, respectively). The primers P6 and P7 contained overlapping complementary sequences that incorporated *Nhe*I sites at the 3′ and 5′ ends of the P5–P6 and P7–P8 primer pair products, respectively. To assemble the gEAm480 truncation plasmid, the P5–P6 and P7–P8 products were digested with *Eco*RI-*Nhe*I and *Nhe*I-*Hin*dIII enzymes, respectively, and cloned into the appropriate sites of pKS02-02 separately, resulting in pBHV-5gE5′ 1 to 480 and pBHV-5gE3′ 480 to 598, respectively. pBHV-5gE3′ 480 to 598 was then digested with *Nhe*I and *Hin*dIII, and the 1.151-kb fragment was gel purified and ligated to *Nhe*I- and *Hin*dIII-digested pBHV-5gE5′ 1 to 480 DNA. In the resulting clone, BHV-5gE Am480, the amino acid 480 (Glutamine) of the protein was replaced with a stop codon. The gE Amber 480 mutation site was flanked on the left by gE ORF (1419 bp) plus gE upstream (950 bp with respect to the gE start codon) and on the right by gE carboxy terminal (343 bp) plus gE downstream (805 bp with respect to the gE stop codon) sequences required for recombination.

To introduce the engineered mutation into BHV-5, full-length gE-deleted GFP expressing BHV-5 constructed earlier, and linearized pBHV-5Am480 DNA were cotransfected into MDBK cells as described earlier (Chowdhury et al., 1999). Non-fluorescent plaques were plaque purified, and the expression of mutant BHV-5gE Am480 was verified by immunoblotting with goat polyclonal antibodies specific for the amino acids 124 to 329 of BHV-5gE ectodomain as developed earlier.

Construction of BHV-5gEAm480-rescued virus

To generate the BHV-5gEAm480-rescued virus (BHV-5gEAm480R), plasmid pBHV-5gE5′3′ DNA (containing the entire *gE* gene and its flanking gI and Us9 sequences) constructed earlier (Chowdhury et al., 2006) was linearized and cotransfected with full-length BHV-5gEAm480 virus DNA. Plaques were randomly picked and analyzed first by *Nhe*I digestion of a PCR-amplified fragment from infected cell DNA (Al-Mubarak et al., 2004). Primer pairs P9 (located within the gE coding region and 248 bp upstream of gE residue 480) and P10 (located within the Us9 coding region and 590 bp downstream of gE residue 480) (Table 3) generated a 840-bp fragment spanning 248 bp upstream to 590 bp downstream of gE residue 480. Several BHV-5gEAm480R viruses lacking the *Nhe*I site were analyzed by immunoblotting, and one of them was selected for further study.

Preparation of radiolabeled mock- and virus-infected cell lysates and immunoprecipitation

Steady-state and pulse-chase labeling were performed as described earlier (Chowdhury et al., 2000). Radioactively labeled infected cell lysates were immunoprecipitated with gE-specific polyclonal goat serum and analyzed by SDS-PAGE (Chowdhury et al., 2000).

Glycopeptidase F and endoglycosidase H digestion

Glycopeptidase F (GlycoF) and endoglycosidase H (EndoH) digestion were performed as described earlier (Al-Mubarak et al., 2004). The digested samples were subjected to SDS-PAGE, and labeled proteins were visualized via autoradiography.

Preparation of ^{32}P radiolabeled mock- and virus-infected cell lysates

For phosphate labeling, confluent MDBK cells were infected with 5 PFU/cell of wild type BHV-5 and BHV-5gEAm480. At 4 h post-infection, the complete growth medium was replaced with serum-free and phosphate-deficient medium (Cellgro by Mediatech, Inc.). At 6 hpi, [^{32}P] ortho-phosphate (100 μCi/ml; Amersham) and 1% FBS were added. Cells were harvested 16 h post-infection and processed for immunoprecipitation as described earlier. Radioactively labeled infected cell lysates were immunoprecipitated with gE-specific goat and/or rabbit antibody described earlier.

Indirect immunofluorescence endocytosis assay

MDBK cells grown on chamber slides were infected with BHV-5 Am480 and wild-type BHV-5 at a multiplicity of infection (MOI) of 5. At 8 h post-infection, the cells were incubated with goat anti-gE-specific antibody for 30 min at 4 °C. After washing (PBS–0.05% Tween-20; PBST), cells were fixed immediately for the 0-min samples (2.5% methanol-free formaldehyde diluted in PBS for 20 min at room temperature) or further incubated for 15 or 30 min at 37 °C and then fixed. After permeabilization (PBS–0.1% triton X-100 for 30 min) and blocking (5% bovine serum albumin in PBS-T for 1 h at 37 °C), they were incubated with Alexa 488-conjugated donkey anti-goat IgG (Molecular Probes). The slides were then cover slipped with gel/mount (Biomeda Corp, Foster City, CA). The cellular localization of gE was analyzed by confocal microscopy.

Animal experiments

Four-week-old New Zealand white rabbits weighing 500 to 600 g (Myrtles Rabbitry, Thomoson Station, TN) were used.

Rabbits were maintained in laboratory isolation cages in our vivarium throughout the experiment, with food and water freely available. All procedures were approved by Kansas State University Animal Care and Use Committee.

A rabbit seizure model described previously (Chowdhury et al., 1997) was used to compare the neuropathogenic properties of BHV-5gEAm480 and BHV-5gEAm480R viruses. Unless otherwise mentioned, 2×10^7 PFU of virus were inoculated per nostril. After infection, the rabbits were observed four times per day for the appearance of neurological symptoms. Nasal swab samples were obtained on day 3 post-infection for virus isolation. For the virus isolation study, rabbits were euthanized at 12 days post-infection or when they showed severe neurological signs. The brains were sectioned and processed as described earlier (Chowdhury et al., 1997).

To compare the neural spread of different viruses, the rabbits were inoculated with each virus as described earlier. Rabbits were euthanized at 11 days post-infection or when the animals showed severe neurological signs, as described previously (Lee et al., 1999). The brain was processed for immunohistochemistry as described earlier (Lee et al., 1999).

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